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STIMULATORY AND INHIBITORY REGULATION OF MYOCARDIAL ADENYLATE CYCLASE BY 5′-GUANYLYL-IMIDODIPHOSPHATE*

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Abstract—Particulate and soluble rat myocardial adenylate cyclase enzymes were characterized with respect to their stimulatory and inhibitory regulation by Gpp(NH)p. Gpp(NH)p (60 μM) stimulated Mg^{2+}- and Mn^{2+}-dependent adenylate cyclase. High concentrations of Gpp(NH)p (600 μM) attenuated the maximal stimulatory response to Gpp(NH)p but only at low cation concentrations. The attenuating effects of 600 μM Gpp(NH)p resulted predominantly from the introduction of a prolonged lag in the kinetics of activation of adenylate cyclase. Steady-state rates of adenylate cyclase activities were similar with either 60 or 600 μM Gpp(NH)p. At any concentration of Gpp(NH)p, the lag was eliminated by Mg ions or isoproterenol. No antihysteretic property for free Mn ions was evident. Forskolin-sensitive particulate adenylate cyclase was not stimulated further by Gpp(NH)p. A 600 μM concentration of Gpp(NH)p inhibited particulate forskolin-sensitive adenylate cyclase at low Mg ion concentrations. In contrast, Gpp(NH)p at 60 μM consistently activated forskolin-sensitive adenylate cyclase after solubilization. The early transient inhibitory properties of 600 μM Gpp(NH)p which resulted in attenuation of adenylate cyclase by 600 μM Gpp(NH)p were diminished by detergent extraction, resulting in only a minor effect of 600 μM Gpp(NH)p to inhibit solubilized adenylate cyclase. These findings indicate that guanine nucleotides exert both stimulatory and inhibitory control upon the myocardial adenylate cyclase enzyme; that solubilization shifts the balance between the stimulatory and inhibitory properties of Gpp(NH)p to allow more dominant expression of the stimulatory response; and that Mg ions critically modify the nature of the myocardial adenylate cyclase response to Gpp(NH)p.

Guanine nucleotides pivotally regulate adenylate cyclase activity [1]. In various tissues, GTP-dependent stimulation or inhibition of adenylate cyclase by hormone reflects the actions of GTP at distinct GTP binding proteins termed N_s and N_i [2]. These guanine nucleotide binding proteins function as transducers of information between the agonist–receptor complex and catalytic adenylate cyclase. The structural and functional properties of both N_s and N_i, responsible for stimulatory and inhibitory control of adenylate cyclase respectively, have been under intense investigation in recent years [3–7].

Myocardial adenylate cyclase is also regulated by guanine nucleotides. Only some studies, however, specifically examined the precise nature of this regulatory control in the heart [8–12]. We have previously characterized cation regulation of particulate and solubilized rat heart adenylate cyclase activity [13]. The present study focuses upon the regulation of myocardial adenylate cyclase by the nonhydrolyzable analogue of GTP, 5′-guanylyl-imidodiphosphate [Gpp(NH)p]. The data support a model which defines guanine nucleotides and magnesium as critical endogenous regulators of adenylate cyclase responsiveness in the heart.

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METHODS

All methods utilized in this paper including preparation of rat myocardial membranes, the adenylate cyclase assay and data analysis have been described in detail elsewhere [13]. Unless otherwise indicated, the incubation mixture contained Tris (0.05 M, pH 7.5), ATP (0.143 mM), an ATP-regenerating system (creatine phosphate, 10 μg; creatine phosphokinase, 14 μg), theophylline (8 mM), KCl (10 mM), [α-^{32}P]ATP ($1–2 \times 10^6$ cpm/assay tube) and 20–25 μg of membrane protein. In each experiment, either $MgCl_2$ or $MnCl_2$ was added to the reaction mixture to achieve the indicated Mg or Mn ion concentrations. Other test agents were used in amounts noted in the figure legends. The final assay volume was 75 μl. All assays were performed for 15 min at 37° and are expressed as nmoles/cyclic AMP (cAMP)/mg protein/15 min unless otherwise indicated. Isolation of [^{32}P]cAMP was accomplished by sequential Dowex and Alumina chromatography using [^{3}H]cAMP as a recovery marker as previously described [13]. In the analysis of the data, the lag interval associated with activation of adenylate cyclase by Gpp(NH)p is defined as the length of the inhibitory phase introduced by Gpp(NH)p prior to achieving a constant steady-state rate of activation of adenylate cyclase.

Solubilization of myocardial adenylate cyclase was accomplished by incubation of rat myocardial membranes with Lubrol PX (0.035% final concentration) at 4° for 60 min with vigorous vortexing every 5 min. Membranes were centrifuged for 90 min at 200,000 *g* and the resulting supernatant fraction containing the solubilized adenylate cyclase activity was assayed directly or stored at −80° for subsequent use. Adenylate cyclase activity prepared and stored in this manner is stable for at least 3 weeks. The pellet remaining after solubilization contained 50% of the starting membrane protein but no enzyme activity when tested with Mg or Mn ions alone or in combination with forskolin. Calculations of solubilized adenylate cyclase activity are based upon the amount of protein in the assay.

RESULTS

We have demonstrated previously that myocardial adenylate cyclase activity is responsive to the stimulatory effects of Gpp(NH)p [13]. In the presence of either Mg or Mn ions, Gpp(NH)p at 100 μM is associated with a maximal stimulatory response. Higher concentrations of Gpp(NH)p result in less stimulation of adenylate cyclase activity when the divalent cation concentration is low. The distinct effects of a maximally stimulatory concentration of Gpp(NH)p (60 μM) and a 10-fold higher concentration of Gpp(NH)p (600 μM) upon Mg^{2+}-dependent adenylate cyclase activity are compared in Fig. 1. Over the entire range of Mg^{2+} concentrations, 60 μM Gpp(NH)p was responsible for an approximate 3-fold increase in adenylate cyclase activity over maximal enzyme activity with Mg^{2+} alone. The K_a for Mg^{2+} as an activator of adenylate cyclase (1.5 ± 0.2 mM) was not changed by Gpp(NH)p (1.3 ± 0.4 mM, $P > 0.5$, $N = 4$). When the higher concentration of Gpp(NH)p was used, along with a maximal Mg ion concentration (>10 mM), stimulation of adenylate cyclase was equivalent to that induced by the lower concentrations of Gpp(NH)p. However, as the Mg ion concentration was lowered from 10 to 0.1 mM, 600 μM Gpp(NH)p was progressively less able to stimulate adenylate cyclase than was 60 μM Gpp(NH)p. Ultimately, at 0.1 mM Mg^{2+}, 600 μM Gpp(NH)p was completely unable to increase adenylate cyclase activity over the activity observed with Mg^{2+} alone.

Kinetic analyses of adenylate cyclase activation provide insight into the characteristics of enzyme regulation by 600 μM Gpp(NH)p, not evident when adenylate cyclase is assayed at a fixed time point. In the presence of 60 or 600 μM Gpp(NH)p, basal Mg^{2+}-dependent adenylate cyclase activity was associated with a finite interval of time before which adenylate cyclase activity achieved a steady state. At a low Mg ion concentration (0.6 mM), this lag associated with activation by 600 μM Gpp(NH)p (8.8 ± 1.1 min) was approximately twice as long as the lag observed with 60 μM Gpp(NH)p (4.7 ± 0.5 min, $P < 0.02$, $N = 4$, Fig. 2, panel A). The differential effects of 600 μM Gpp(NH)p were confined predominantly to the early time points. The steady-state rates of cAMP production (after the initial lag periods) were similar with 60 or 600 μM Gpp(NH)p. At either Gpp(NH)p concentration, the lag interval was progressively eliminated by increasing the Mg ion concentration (Fig. 2, panels B and C).

The effects of 60 μM Gpp(NH)p and 600 μM Gpp(NH)p on adenylate cyclase activity in the presence of Mn^{2+} can similarly be distinguished from each other (Fig. 3). Focusing upon a range of Mn ion concentrations associated exclusively with progressive stimulation of adenylate cyclase (0.01 to 0.6 mM), a consistently stimulatory concentration of Gpp(NH)p (60 μM) enhanced enzyme activity at all of these Mn ion concentrations. A 2- to 3-fold stimulation of Mn^{2+}-dependent adenylate cyclase activity without an alteration in the sensitivity of the adenylate cyclase complex for Mn^{2+} paralleled the effects of

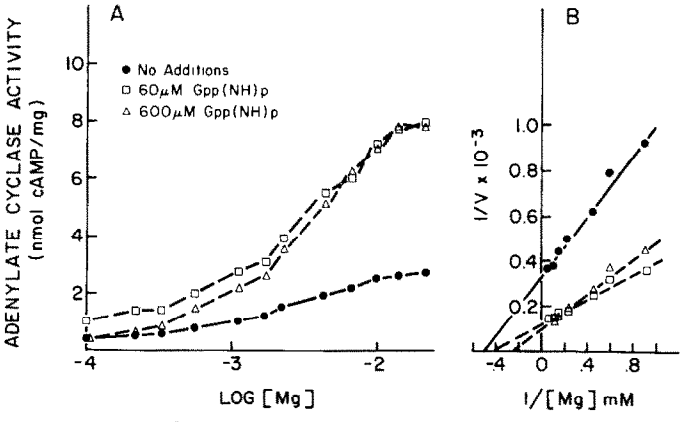

Fig. 1. Effect of Gpp(NH)p on Mg^{2+} activation of particulate adenylate cyclase. (A) Adenylate cyclase activity was measured in the presence of 0 (●), 60 μM (□) or 600 μM (△) Gpp(NH)p at the indicated Mg ion concentrations as described in Methods. (B) Double-reciprocal plot transformation of the data represented in panel A. For panel B, adenylate cyclase activity (V) is expressed as pmoles cAMP/mg protein/15 min. The experiment shown is representative of three separate experiments, each performed in triplicate.

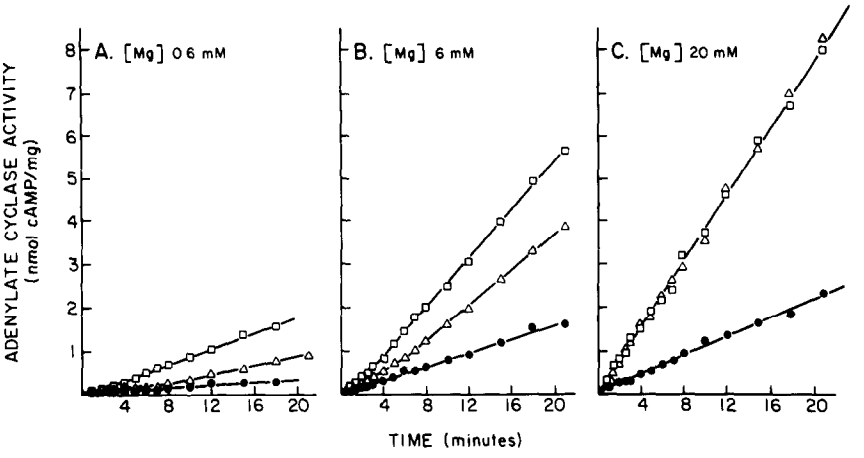

Fig. 2. Kinetics of activation of Mg^{2+}-dependent particulate adenylate cyclase in the presence of 60 or 600 μM Gpp(NH)p. Adenylate cyclase activity was initiated by the addition of prewarmed rat myocardial membranes to tubes containing reaction mix and 0 (●), 60 μM (□) or 600 μM (△) Gpp(NH)p at various Mg ion concentrations. At the indicated time points, aliquots were withdrawn for determination of cAMP as described in Methods. The experiment shown is representative of four separate experiments, each performed in duplicate.

60 μM Gpp(NH)p upon Mg^{2+}-dependent adenylate cyclase. A higher concentration of Gpp(NH)p (600 μM) presented a different profile of adenylate cyclase activation. Adenylate cyclase activity was refractory to stimulation by 600 μM Gpp(NH)p at Mn ion concentrations below 0.1 mM. Ineffective stimulation of adenylate cyclase by 600 μM Gpp(NH)p was characteristic of low concentrations of either divalent cation (Mg^{2+} or Mn^{2+}). As the Mn ion concentration was increased, a window of Mn ion concentrations (0.2 to 0.6 mM) allowing progressive stimulation of adenylate cyclase by 600 μM Gpp(NH)p was observed. Higher Mn ion concentrations (>0.6 mM) which directly attenuate adenylate cyclase, rendered the membrane refractory to stimulation by Gpp(NH)p.

In this setting also, a kinetic analysis of the time course of Mn^{2+}-dependent adenylate cyclase activity in the presence of varied Gpp(NH)p concentrations was revealing (Fig. 4). Stimulation of adenylate cyclase by 60 μM Gpp(NH)p was associated with a lag interval of approximately 5 min over a 10-fold range of Mn ion concentrations (0.06 to 0.6 mM). Increasing Mn^{2+} increased the initial and terminal rates of cAMP production but was not associated with an alteration in the lag period *per se*. Similarly,

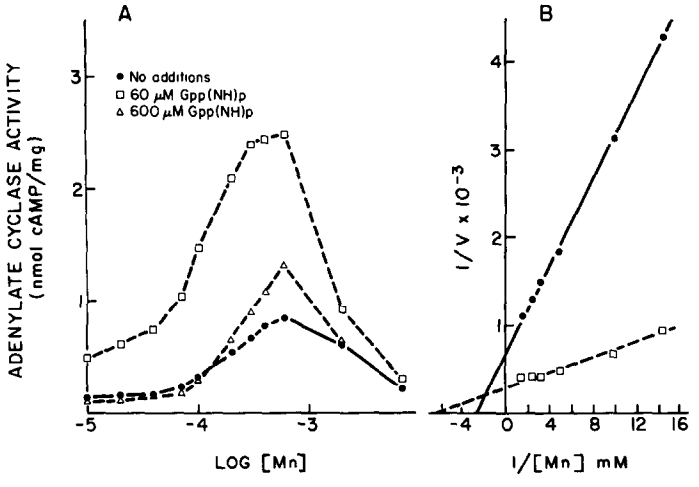

Fig. 3. Effect of Gpp(NH)p on Mn^{2+} activation of particulate adenylate cyclase. (A) Adenylate cyclase activity was measured in the presence of 0 (●), 60 μM (□) or 600 μM (△) Gpp(NH)p at the indicated Mn ion concentrations as described in Methods. (B) Double-reciprocal plot of adenylate cyclase activity in the presence of 0 (●) or 60 μM (□) Gpp(NH)p as a function of Mg ion. For panel B, adenylate cyclase activity (V) is expressed as pmoles cAMP/mg protein/15 min. A small decrease in the K_a for Mn^{2+} with 60 μM Gpp(NH)p was not significant in three separate experiments.

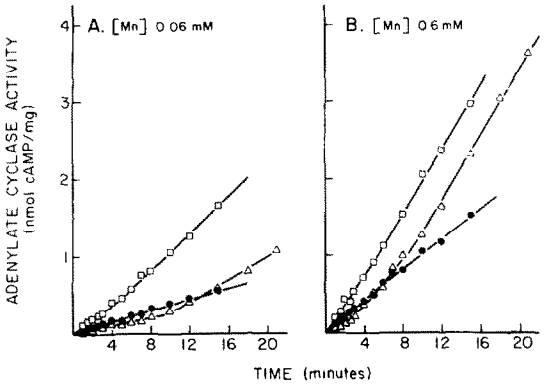

Fig. 4. Kinetics of activation of particulate Mn^{2+}-dependent adenylate cyclase in the presence of 60 or 600 μM Gpp(NH)p. Adenylate cyclase activity was initiated by addition of prewarmed rat myocardial membranes to tubes containing reaction mix and 0 (●), 60 μM (□) or 600 μM (△) Gpp(NH)p at 0.06 mM (panel A) or 0.6 mM (panel B) Mn^{2+}. At the indicated time points, aliquots were withdrawn for determination of cAMP. The experiment shown is representative of four separate experiments, each performed in duplicate.

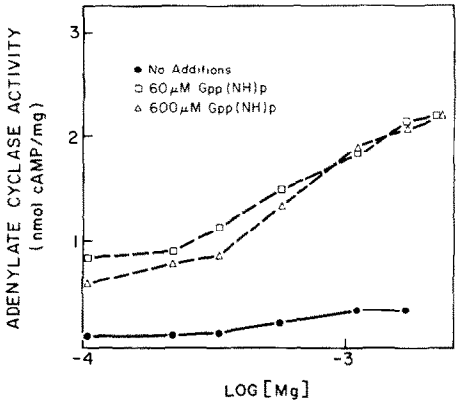

Fig. 5. Effect of Gpp(NH)p on Mg^{2+} activation of solubilized adenylate cyclase. Adenylate cyclase activity was measured in the presence of 0 (●), 60 μM (□), or 600 μM (△) Gpp(NH)p at the indicated Mg ion concentrations as described in Methods. The experiment shown is representative of three separate experiments, each performed in triplicate.

stimulation of adenylate cyclase by 600 μM Gpp(NH)p occurred with a lag period not changed by the Mn ion concentration. With the higher concentration of Gpp(NH)p, however, the lag interval associated with activation of adenylate cyclase was twice as long as the lag associated with 60 μM Gpp(NH)p (10 min vs. 5 min). The modulatory influence of a high concentration of Gpp(NH)p at early time points to prolong the interval before steady-state adenylate cyclase activation was achieved was similar to the effects of 600 μM Gpp(NH)p at a low Mg ion concentration (Fig. 2, panel A). However, no antihysteretic property for free Mn ions upon Gpp(NH)p-dependent basal adenylate cyclase was observed. At either Mn ion concentration, the lag interval associated with stimulation of basal adenylate cyclase by Gpp(NH)p was progressively eliminated by Mg ions (data not shown).

An early effect unique to 600 μM Gpp(NH)p to inhibit basal adenylate cyclase activity in the presence of Mn^{2+} was observed consistently. This profound inhibitory property of 600 μM Gpp(NH)p during the initial 10 min was followed by a steady-state rate of cAMP production which exceeded the basal rate. Compared to basal adenylate cyclase or adenylate cyclase activity in the presence of 60 μM Gpp(NH)p, 600 μM Gpp(NH)p-activated enzyme activity remained linear for a considerably longer period (at least another 20 min). These effects of 600 μM Gpp(NH)p parallel observations in other systems of prolonged activation of adenylate cyclase by Gpp(NH)p [14].

Previous studies utilizing a solubilized preparation of myocardial adenylate cyclase suggested that during detergent extraction, the catalytic unit of adenylate cyclase is released from certain constraints in its membrane bound form [13]. We reasoned that the solubilized preparation might provide a simpler model in which the structural requirements for modulation by 60 and 600 μM Gpp(NH)p could be defined better. The effect of Gpp(NH)p upon solubilized Mg^{2+}-dependent adenylate cyclase is depicted in Fig. 5. Mg ions alone ineffectively stimulated solubilized myocardial adenylate cyclase. However, both 60 and 600 μM Gpp(NH)p enhanced the agonist property of free Mg ions. Gpp(NH)p at 60 μM augmented Mg^{2+}-dependent adenylate cyclase approximately 3- to 5-fold. The adenylate cyclase response of the solubilized enzyme to 600 μM Gpp(NH)p in the presence of Mg^{2+} provided a marked contrast to the observations in particulate membranes (compare Figs. 1 and 5). In particulate adenylate cyclase preparations, 600 μM Gpp(NH)p stimulated enzyme activity as effectively as 60 μM Gpp(NH)p at high Mg^{2+} but did not augment basal adenylate cyclase activity at very low Mg^{2+}. After detergent extraction, 600 μM Gpp(NH)p stimulated adenylate cyclase at all Mg ion concentrations tested. The attenuating effect of high concentrations of Gpp(NH)p (600 μM) as compared to lower Gpp(NH)p concentrations, at low Mg ion concentrations, was now virtually nonexistent following detergent extraction.

In separate experiments, the effects of detergent extraction upon the time course of activation of solubilized adenylate cyclase by 60 and 600 μM Gpp(NH)p were defined. The hysteretic lag associated with activation of adenylate cyclase by 600 μM Gpp(NH)p was diminished by solubilization, resulting in similar lags associated with activation of adenylate cyclase by either 60 or 600 μM Gpp(NH)p (Fig. 6). One effect of detergent extraction, then, appears to be a marked diminution of the early transient inhibitory properties of a high concentration of Gpp(NH)p. This effect of detergent extraction to alter the characteristics of activation of adenylate cyclase by 600 μM Gpp(NH)p constitutes further evidence that the inhibitory action of 600 μM Gpp(NH)p result from a specific interaction of the guanine nucleotide with a determinant on the adenyl-

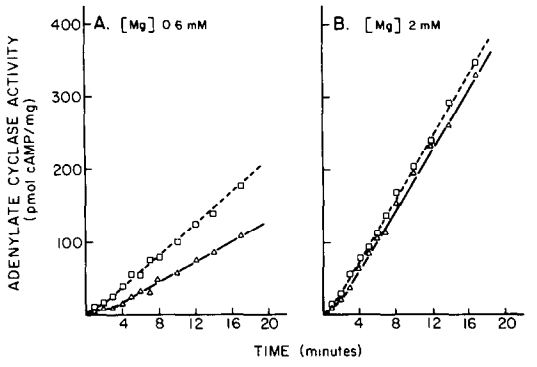

Fig. 6. Kinetics of activation of solubilized Mg^{2+}-dependent adenylate cyclase by 60 μM and 600 μM Gpp(NH)p. Adenylate cyclase activity was initiated by addition of prewarmed solubilized rat myocardial membranes to tubes containing reaction mix and 60 μM (□) or 600 μM (△) Gpp(NH)p at 0.6 mM (panel A) or 2 mM (panel B) Mg^{2+}. At the indicated time points, aliquots were withdrawn for determination of cAMP as described in Methods. The experiment shown is representative of three separate experiments, each performed in duplicate.

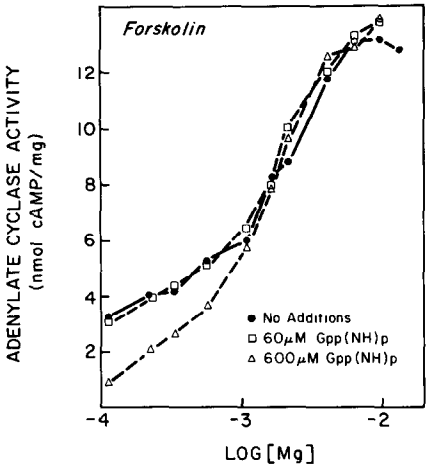

Fig. 7. Effect of Gpp(NH)p on forskolin-activated Mg^{2+}-dependent particulate adenylate cyclase activity. Adenylate cyclase activity was measured in the presence of forskolin (170 μM) and 0 (●), 60 μM (□), and 600 μM (△) Gpp(NH)p at the indicated Mg ion concentrations as described in Methods. The experiment shown is representative of four separate experiments, each performed in triplicate.

ate cyclase complex rendered less effective by solubilization.

Another approach to the examination of the dual properties of the guanine nucleotides to activate and inhibit cardiac adenylate cyclase took advantage of the agonist forskolin [15, 16]. One action of forskolin is believed to involve a direct effect at the catalytic unit of adenylate cyclase. Although stimulation of adenylate cyclase by forskolin does not require guanine nucleotides or the guanine nucleotide binding regulatory proteins, it has now been amply demonstrated that N_s or N_i can modulate forskolin-sensitive adenylate cyclase activity [17–20]. Less than additive stimulatory effects of Gpp(NH)p and forskolin characterize adenylate cyclase systems that are dually regulated by guanine nucleotides at N_s and N_i [21]. Accordingly, we examined the effects of 60 or 600 μM Gpp(NH)p upon forskolin-sensitive adenylate cyclase in membrane bound and solubilized preparations of rat heart adenylate cyclase. If the effect of detergent extraction to diminish the transient inhibitory property of 600 μM Gpp(NH)p relates to the loss of an inhibitory guanine nucleotide binding regulatory protein, one might expect the response of forskolin-sensitive adenylate cyclase to Gpp(NH)p to be augmented after solubilization.

The effects of Gpp(NH)p upon forskolin-sensitive particulate adenylate cyclase are depicted in Fig. 7. Over a range of Mg ion concentrations, forskolin-sensitive particulate adenylate cyclase was not altered by Gpp(NH)p at concentrations <60 μM (only data at 60 μM are shown). It was inhibited however, by 600 μM Gpp(NH)p at Mg ion concentrations below 1 mM. Inhibition of forskolin-sensitive adenylate cyclase by 600 μM Gpp(NH)p resulted primarily from an initial lag period. The ultimate steady-state rates of activation in the presence of forskolin alone or forskolin and 600 μM Gpp(NH)p were similar (Fig. 9, panel B). Higher Mg ion concentrations progressively eliminated the Gpp(NH)p-induced transient inhibitory phase, thereby reversing the inhibitory properties of 600 μM Gpp(NH)p (data not shown).

In separate studies, we characterized the effect of 600 μM Gpp(NH)p upon Mn^{2+}-dependent forskolin-sensitive adenylate cyclase. Stimulation by 600 μM Gpp(NH)p was associated with a lag interval of approximately 8 min (Table 1). The length of this lag interval remained constant despite a 40-fold range of Mn ion concentrations. In a previous study we demonstrated that Mg^{2+} (at similar concentrations) shortens the lag interval associated with stimulation of forskolin-sensitive adenylate cyclase by 600 μM Gpp(NH)p [13]. Antihysteretic properties for Mg^{2+}, but not for Mn^{2+}, in the presence or absence of forskolin (Figs. 2 and 4), constitute important evidence that the lag associated with 600 μM Gpp(NH)p does not result from nonspecific sequestration of divalent cations *per se*, but relates to a distinct inhibitory property of Gpp(NH)p at the adenylate cyclase complex. In fact, a second difference between 600 μM Gpp(NH)p-stimulated forskolin-sensitive adenylate cyclase in the presence of Mg^{2+} or Mn^{2+} was evident. In the presence of Mg^{2+}, the sole effect of 600 μM Gpp(NH)p upon forskolin-sensitive adenylate cyclase was to introduce an initial Mg^{2+}-sensitive inhibitory lag. The ultimate steady-state rate of activation was not altered. In contrast, in the presence of Mn^{2+}, in addition to introducing an initial Mn^{2+}-insensitive inhibitory lag, 600 μM Gpp(NH)p modulated the ultimate steady-state rate of activation of forskolin-sensitive adenylate cyclase (Table 1).

Solubilization markedly altered the response of forskolin-sensitive Mg^{2+}-dependent adenylate cyclase to Gpp(NH)p. In contrast to particulate forskolin-sensitive adenylate cyclase which was not further stimulated by Gpp(NH)p (Fig. 7), the det-

Table 1. Modulation of forskolin-sensitive adenylate cyclase activity by 600 μM Gpp(NH)p

Mn (mM)	Adenylate cyclase activity (pmoles cAMP/mg protein/min) (−) Gpp(NH)p	(+) Gpp(NH)p	Lag introduced by Gpp(NH)p (min)
0.01	87	24	8.5
0.07	160	84	7.7
0.1	167	96	7.5
0.4	198	243	7.5

Time courses were carried out as described in Methods. Gpp(NH)p-dependent adenylate cyclase activity reported is the secondary rate which followed the initial inhibitory lag. Results represent the average of two to three time courses performed in duplicate at each Mn ion concentration.

ergent-extracted forskolin-stimulated enzyme was further stimulated by 60 μM Gpp(NH)p over the entire range of Mg ion concentrations (0.1 to 10 mM) (Fig. 8). In fact, the response to 60 μM Gpp(NH)p and forskolin was additive in the solubilized enzyme (data not shown). Responsiveness to the stimulatory actions of 60 μM Gpp(NH)p in detergent-extracted but not the particulate adenylate cyclase preparation could result from either the acquisition of a stimulatory mechanism or (more likely) loss of an inhibitory mechanism during solubilization.

We next examined the solubilized adenylate cyclase response in the presence of a higher concentration of Gpp(NH)p associated with a more dominantly inhibitory effect. At a maximal Mg ion concentration (10 mM), both 60 and 600 μM Gpp(NH)p stimulated forskolin-sensitive solubilized adenylate cyclase equivalently. At lower Mg ion concentrations (<1 mM) 600 μM Gpp(NH)p became progressively less stimulatory than 60 μM Gpp(NH)p. Ultimately, at a very low Mg ion concentration, 600 μM Gpp(NH)p frankly inhibited forskolin-sensitive solubilized adenylate cyclase. Thus, when compared to the response of forskolin-sensitive particulate adenylate cyclase to Gpp(NH)p which occurs only at limited experimental conditions and is solely inhibitory in nature, the response of the forskolin-sensitive solubilized enzyme to 600 μM Gpp(NH)p was more elaborate. A 600 μM concentration of Gpp(NH)p either stimulated (at high Mg^{2+}) or slightly inhibited (at low Mg^{2+}) forskolin-sensitive adenylate cyclase. Detergent extraction altered the balance diminishing the inhibitory properties and, thus, allowed the stimulatory properties of 600 μM Gpp(NH)p upon forskolin-sensitive adenylate cyclase to dominate.

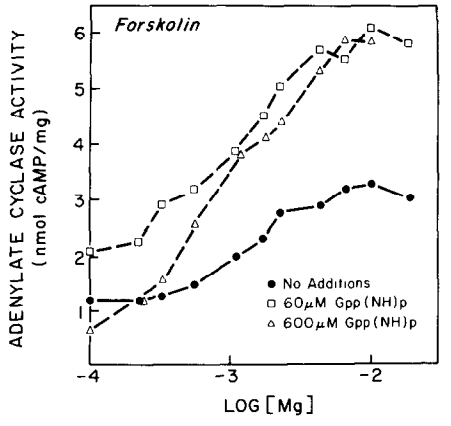

Fig. 8. Effect of Gpp(NH)p on forskolin-sensitive Mg^{2+}-dependent solubilized adenylate cyclase activity. Adenylate cyclase activity was measured in the presence of forskolin (170 μM) and 0 (●), 60 μM (□), or 600 μM (△) Gpp(NH)p at the indicated Mg ion concentrations as described in Methods. The experiment shown is representative of three separate experiments, each performed in triplicate.

Hormones are widely recognized to be antihysteretic agents [14, 22, 23]. Therefore, we next evaluated whether the effect of saturating Mg^{2+} to accelerate the activation of adenylate cyclase by guanine nucleotides was mimicked by hormonal stimulation. The beta-adrenergic receptor agonist, isoproterenol, totally eliminated the initial lag associated with activation of Mg^{2+}- or Mn^{2+}-dependent adenylate cyclase by either 60 or 600 μM Gpp(NH)p (Fig. 9). The effect of isoproterenol was confined to reversal of the initial lag period. At either concentration of Gpp(NH)p, the steady-state rate of cAMP production in the presence of isoproterenol and Gpp(NH)p was similar to that observed with Gpp(NH)p alone. Similarly, the transient inhibitory phase induced by 600 μM Gpp(NH)p upon forskolin-sensitive adenylate cyclase was eliminated by isoproterenol. These antihysteretic effects of isoproterenol were prevented by the simultaneous presence of the beta-adrenergic antagonist propranolol (data not shown). The antihysteretic properties of isoproterenol constitute further evidence that the inhibitory effects of 600 μM Gpp(NH)p result from a direct interaction of Gpp(NH)p with the adenylate cyclase enzyme complex.

DISCUSSION

The data presented in this paper delineate the dual actions of Gpp(NH)p as a stimulatory and inhibitory regulator of myocardial adenylate cyclase. Although the stimulatory property of guanine nucleotides upon myocardial tissue was observed a decade ago [8], the characteristics of guanine nucleotide dependent

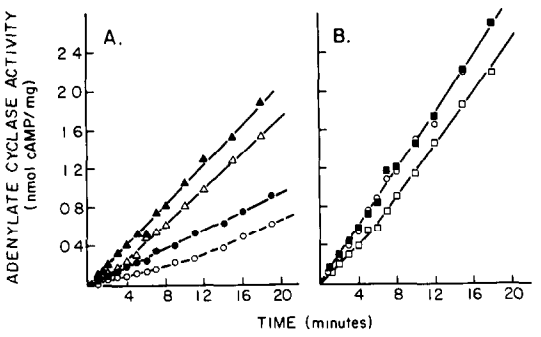

Fig. 9. Effect of isoproterenol to eliminate the lag associated with activation of adenylate cyclase by 60 or 600 μM Gpp(NH)p. (A) Adenylate cyclase was initiated by the addition of prewarmed rat myocardial membranes to tubes containing reaction mix (1 mM Mg^{2+}) and 60 μM (△, ▲) or 600 μM (○, ●) Gpp(NH)p in the presence (▲, ●) or absence (△, ○) of isoproterenol (1 μM). (B) Prewarmed membranes were added to tubes containing reaction mix (1 mM Mg^{2+}) and forskolin (170 μM) alone (○), in the presence of 600 μM Gpp(NH)p (□), or in the presence of 600 μM Gpp(NH)p and 1 μM isoproterenol (■). For A and B, at the indicated time points, aliquots were withdrawn for determination of cAMP as described in Methods. The experiment shown is representative of four (A) or five (B) separate experiments, each performed in duplicate.

inhibition of adenylate cyclase in the heart have received little attention. In an experimental design that utilized particulate and solubilized forms of rat heart membranes and a kinetic approach to the adenylate cyclase assay, we were able to distinguish Gpp(NH)p as both an agonist and antagonist of adenylate cyclase. A distinct property of high concentrations of Gpp(NH)p to inhibit adenylate cyclase was evident. The effect of high concentrations of Gpp(NH)p to diminish adenylate cyclase activity was not due to contamination of Gpp(NH)p with other guanine nucleotides (particularly GDP; [13]) nor merely the result of a nonspecific sequestration of cations resulting in decreased availability of free divalent cations as allosteric regulators. In fact, we have recently observed an inhibitory property at similar concentrations of the naturally occurring guanine nucleotide, GTP. Inhibition of adenylate cyclase by 600 μM Gpp(NH)p is best accounted for by the introduction of a discrete lag period in the onset of activation. The lag, which was much less evident at lower concentrations of Gpp(NH)p, was completely overcome by Mg^{2+} or isoproterenol. These findings are compatible with an effect of Gpp(NH)p at both inhibitory and stimulatory GTP-binding proteins, N_i and N_s. In separate studies, we have documented the presence of these regulatory units in the heart [24]. If Gpp(NH)p activated both inhibitory and stimulatory regulatory subunits simultaneously, the resulting adenylate cyclase activity would reflect the concentration–response characteristics of Gpp(NH)p at each site as well as a variety of other factors including the characteristics of the membrane preparation itself (i.e. the amount of N_s and N_i, effectiveness of their coupling to the catalytic moiety) and the conditions of the assay incubation (i.e. divalent cations, forskolin). In fact, studies in the detergent-extracted enzyme illustrate that the net effect of Gpp(NH)p (stimulatory or inhibitory) may reflect a balance between the functional linkage of either subunit to catalytic adenylate cyclase. Whereas high concentrations of Gpp(NH)p were inhibitory in membrane preparations, they became more stimulatory in solubilized preparations under the same conditions. A stimulatory response to guanine nucleotides following solubilization has been described in a variety of adenylate cyclase systems [8, 9, 11, 25, 26]. Our observations indicate that only a minor inhibitory effect of 600 μM Gpp(NH)p is retained after solubilization. This small inhibitory effect of Gpp(NH)p is presumed to reflect an action at N_i, which may be functionally present at low levels in the solubilized myocardial adenylate cyclase preparation. However, direct support for the notion that the inhibitory effects of Gpp(NH)p in particulate and solubilized myocardial adenylate cyclase are mediated by N_i awaits the quantitative assessment of N_i in these two preparations as well as the demonstration that inhibition by Gpp(NH)p is abolished when N_i is totally inactivated by pertussis toxin. Such studies are in progress.

The greater stimulatory properties of high concentrations of Gpp(NH)p after solubilization were further revealed by the forskolin-sensitive enzyme. Forskolin is believed to stimulate adenylate cyclase via two effects: a direct action upon catalytic activity and another effect which is influenced by N_s [17–20]. In the presence of forskolin, the balance of stimulatory and inhibitory properties of Gpp(NH)p appears to favor inhibition by Gpp(NH)p at a low Mg^{2+} concentration [21]. In fact, no independent stimulatory effect of Gpp(NH)p was demonstrated in particulate membranes when forskolin was present. Following solubilization, we observed an impressive effect of Gpp(NH)p to enhance forskolin-sensitive adenylate cyclase. This enhanced expression of forskolin-sensitive soluble enzyme activity in the presence of Gpp(NH)p implies enhanced expression of the stimulatory guanine nucleotide binding regulatory subunit (N_s). There are several molecular mechanisms by which this result could be achieved including actual loss of N_i units, functional uncoupling between N_i and C, better intrinsic coupling between N_s and C, or an effect on a still to be identified component of the regulatory complex. In our previous studies, we observed a loss of the inhibitory properties of high Mn ion concentrations after solubilization [13]. Coincident loss of the inhibitory properties of Gpp(NH)p and high Mn^{2+} may suggest a molecular relationship between the locus of inhibition by Mn^{2+} and Gpp(NH)p or may simply be a fortuitous coincidence and awaits further study with resolved components of the adenylate cyclase complex.

Data in this manuscript support a role for Mg ions (but not Mn ions) in the activation of myocardial adenylate cyclase at the guanine nucleotide-binding regulatory subunit. Similar regulation by Mg^{2+} is observed in adenylate cyclase preparations from other tissues [22, 23]. Specifically, only Mg ions diminished the lag period associated with activation of basal adenylate cyclase by Gpp(NH)p. At a low Mg

ion concentration, inhibition of adenylate cyclase by Gpp(NH)p was more dominantly expressed. At high Mg^{2+}, stimulation by high Gpp(NH)p was observed. These differing effects are specifically a function of the antihysteretic properties of Mg^{2+} to reduce the Gpp(NH)p–associated lag period. The Mg^{2+} requirements for activation of the guanine nucleotide binding regulatory proteins (N_s and N_i) are known to differ. Recent evidence documents a lower Mg ion requirement for activation of N_i (micromolar) than that required for stimulation of N_s (millimolar; [27]). Insofar as a low Mg^{2+} concentration may be inadequate to support activation of N_s and stimulation of adenylate cyclase, it may serve to enhance expression of guanine nucleotide actions at N_i. In this way the Mg ion concentration may critically modulate the balance of functional expression of N_s and N_i.

The data reported here can be evaluated with respect to the cellular concentrations of cations and guanine nucleotides. The K_a for activation of adenylate cyclase by free Mg ions (1.5 mM) was substantially higher than the estimates of intracellular Mg (0.3 to 0.5 mM; [28]). In contrast, the intracellular GTP concentration was estimated to be greater than 100 μM, suggesting that the cellular guanine nucleotide concentration is far in excess of that required for maximal adenylate cyclase activation. Current models regarding the physiologic regulation of adenylate cyclase have, therefore, focused on the action of hormone-occupied receptors to activate N_s by lowering the Mg^{2+} requirement to levels below that found intracellularly, thereby permitting Mg^{2+}-dependent activation to occur [29]. However, certain actions of guanine nucleotides upon the hormone-receptor adenylate cyclase complex can only be observed at substantially higher guanine nucleotide concentrations than those associated with activation of the stimulatory or inhibitory GTP-binding proteins. In particular, modulation of agonist binding to myocardial or hepatic $alpha_1$-adrenergic receptors is maximal only at millimolar Gpp(NH)p [30, 31]. With full awareness that estimates of intracellular divalent cation and guanine nucleotide concentrations are technically difficult and may not reflect possible critical compartmentalization of these species within the cell or at the cell membrane, the data reported in this paper suggest a model in which local concentrations of guanine nucleotides may be regulatory. At the prevailing low intracellular divalent cation concentrations, high intracellular guanine nucleotide levels would appear to serve as inhibitory cofactors. This model is physiologically attractive in that an essential enzyme cofactor, GTP, now becomes a pivotal regulator as well.

The exact sites of metal ion and guanine nucleotide regulation of myocardial adenylate cyclase remain to be directly elucidated. Ultimately, isolation of the individual components of the myocardial adenylate cyclase complex including N_s, N_i and the catalytic unit itself and reconstitution under carefully defined experimental conditions should provide critical insights into the site and nature of the regulatory control of the enzyme by endogenous cofactors.

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